The University of Chicago

GERMINATION BEHAVIOR OF MAGNOLIA GRANDIFLORA

A DISSERTATION SUBMITTED TO THE FACULTY OF THE DIVISION OF THE BIOLOGICAL SCIENCES IN CANDIDACY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF BOTANY
1930

By
CLYTEE R. EVANS

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GERMINATION BEHAVIOR OF MAGNOLIA GRANDIFLORA

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CLYTEE R. EVANS

Introduction

Magnolia grandiflora (L.) belongs to a very old genus now limited to southeastern North America, southern Mexico, and southern and eastern Asia. It is found in the coastal plain of the southeastern part of the United States. Within this area it grows in rich moist soil on the borders of river swamps and ponds, particularly on hammocks in Florida and fertile rolling slopes along the Mississippi River.

In the literature on seed germination there is very little reference to the behavior of the various species of *Magnolia*. MILLAIS (30) mentions the ready germination of *Magnolia macrophylla* if the seeds are sown immediately after being gathered. He refers to the seeds of certain Chinese species as short-lived, being capable of germination for only a very short time after they are shed. He suggests for seeds of *M. grandiflora* the treatment outlined by BAILEY (2). This method calls for storage of the freshly gathered seeds in dry sand until February, then in moist sand for a week or ten days to loosen the outer fleshy coat. After these coats are removed by washing in water, the seeds are sown in a cold frame. TOUMEY (44) found that the seeds of *M. acuminata* give a germination of only about 3 per cent at maturity, although 68 per cent seem sound. He believes the hard endosperm of these seeds requires several months for the absorption of sufficient water for germination. Observations of a botanist in Florida are to the effect that seeds of *M. grandiflora* germinate in moist situations in the shade of the trees in great numbers in the spring following their shedding in the fall. The writer has observed that seeds of this species when shed from the tree germinate very rarely although they are viable. Probably the limits of the natural range may be related to the germination behavior of the seed.

Nurserymen in the region where *Magnolia grandiflora* grows advise macerating the seeds, stratifying them in moist sand at low temperatures until spring, and then planting. They say that if the seeds dry out they will lie over a year, if they come up at all.

The chemical composition of the seed and the changes taking place in the endosperm and embryo during germination have had almost no attention. WEHMER (47) mentions a fat and an ethereal oil, magnoliol, as having been determined in the seed. Alkaloids, glucosides, resin, and saponin have been claimed on evidence not altogether convincing. Studies such as those by TOOLE (43) on maize seeds and ECKERSON (18) on wheat seedlings interested the writer to attempt to study the chemical changes in the seeds of *M. grandiflora* during normal germination under a given set of conditions. It was believed also that these studies might point the way toward a method of overcoming their dormancy.

Many attempts were made to hasten the after-ripening or to overcome dormancy. A wide variety of conditions was used, as will be indicated in the section dealing with experimental work, but without marked success. A summary of the extensive literature which guided the work is not attempted here.

During the studies of early germination behavior, the writer became interested in the oxidation-reduction mechanisms of the embryos and the food reserves. This interest centered in the activity of oxidase and catalase and in the glutathione mechanism. This compound, which HOPKINS (24, 25) discovered and considered a dipeptide of cysteine and glutamic acid, has been reinterpreted by him as a tripeptide including glycine, as well as the two amino acid components first discovered. Since 1921 it has been considered by many workers in animal physiology as having an important rôle in respiration, being present in greatest quantities in tissues having a high metabolic rate. It has been found to be widely distributed in plant tissues also. Up to the present time only slight attention has been given it in connection with studies on seed germination. FIRKERT and COMHAIRE (20) have demonstrated its presence not only in active meristematic regions but also in reserves. Of particular interest is the fact that they studied the increase in glutathione in seeds of the pea during absorption of water. This formation of glutathione

has been attributed by VIVARIO and LE CLOUX (46) to the hydrolytic decomposition of polypeptide complexes. These studies, together with such work as that of KOZLOWSKI (28) on the distribution of non-protein cysteine in meristems and other plant tissues, and that of CAMP (7) on the shift in metabolic activity in flower primordia, determined quantitatively on the basis of the nitroprusside color test for glutathione, made it seem particularly desirable to study the glutathione content of *Magnolia grandiflora* seeds during germination. Moreover, interest in compounds containing -SH groups has been stimulated by work done at the Lankenau Hospital in Philadelphia on the healing properties of such compounds as thioglucose, cysteine, and thioglycollic acid. Certain of these have been found to stimulate growth, actually causing an increase in cell division in root tips. It seemed that these compounds might have some significance in the problem of dormancy, such as is exhibited by *Magnolia* seeds.

This paper presents the results of a study of the behavior of seeds of *M. grandiflora* during the preparation for germination and the early stages of the process, with particular reference to the overcoming of dormancy, to the chemical changes taking place during after-ripening, and to the presence and development of oxidizing mechanisms, especially glutathione.

Materials and method

The seeds used in these experiments were of four lots. Lot 1 was 1928 seed received in the pulp nine months after being gathered. Lots 2, 3, and 4 were all 1929 seed. Lot 3 was gathered by the writer in Mississippi in October, 1929; lot 2 was received approximately two months after being harvested; lot 4, seven months afterward. Some seeds of each lot were stored dry at room temperature in glass jars; some of lots 2, 1, and 4, moist at 10°C.; and some of lot 1 were stored dry at 10°. Lot 4 was infected with a green mold when received. Preliminary to storage in damp sterile sand at 10°, the seeds were macerated, after soaking in water 24 hours, and disinfected in $CaOCl_2$ solution.

STRUCTURE.—Observations were made on the gross structure of the seed and seedling and on the course of development of the latter. These were followed by microscopic and microchemical studies of

freehand sections of which camera lucida drawings were made in many instances.

GERMINATION STUDIES.—All seeds in dry storage were macerated and soaked for two hours in $CaOCl_2$ solution prepared as suggested by WILSON (48). Tests were run using the seed with the lignified coat on, and others with it removed on one side. Embryos free from the endosperm were set to germinate also. The temperatures used were 10°, 20°, 23°, 29°, and an alternation of 10° and 23°C. The many chemicals used were those suggested by the results of other investigations on problems of dormancy or stimulation.

CHEMICAL CHANGES.—Seeds germinated in the jars of moist sand stored at 10° were studied intensively in an attempt to determine particularly the changes taking place between the splitting of the coat and the emergence of the hypocotyl. In many cases these studies were extended to include the dry seeds of the same lot and seedlings older than the two stages just mentioned. The microchemical tests used were largely the ones for reducing sugars, starch, amino acids, reserve proteins, soluble proteins, fats, and a few others given by ECKERSON (16) or TUNMANN (45). Free acids were determined by titration with NaOH, using phenolphthalein as the indicator. Neutral red was used to detect localization of changes in reaction in the endosperm and embryo.

OXIDIZING ENZYMES AND GLUTATHIONE.—For the determination of the activity of oxidase, peroxidase, and catalase, qualitative tests suggested by ECKERSON (16) were used. These were checked further by quantitative determinations of oxidase according to BUNZELL (6) and catalase by the APPLEMAN method (1). In testing for glutathione, the nitroprusside method of HOPKINS as used by FINK (19) was adopted. Sections of the seeds were heated in dilute acetic acid to the boiling point and then placed in shallow dishes in enough saturated ammonium sulphate to cover them well. Immediately a few drops of a 5 per cent solution of sodium nitroprusside $Na_2Fe(CN)_5NO \cdot 2H_2O$ were added and the material allowed to stand for at least five minutes. Then 1 cc. of ammonium hydroxide, full strength, was added. The appearance of pink to permanganate color was considered to indicate the presence of glutathione. The

depth of the color and the length of time it remained indicated the amount present. The glutathione test was run on seeds of *Ricinus* also.

Results

Structure

Diagrams of the structure of the seeds of *Magnolia* have been published by Brandza (3) and Sargent (41). Brandza interprets the fleshy outer coat as the testa; the inner lignified one as the tegmen; and the membranous innermost one as the remnant of the nucellus. Sargent calls the membranous coat the tegmen, and considers the lignified one and the fleshy one as parts of the testa. Brandza's opinion is accepted here. The fleshy outer coat is made up of three regions: a three-layered epidermis, a wide region of at least six rows of large fleshy cells containing much oil, and within this a layer of small cells forming a sort of inner epidermis. This fleshy coat contains 57 per cent oil and a considerable quantity of reducing sugars. It is traversed by the vascular bundle which leads through the raphe to the chalaza, a region where the coats are undiverged. The lignified coat is made up of radial rows of heavy-walled cells. The nucellus is a membranous layer, brown in color and easily plasmolyzed by the many reagents used in the microchemical tests described elsewhere in this paper. The chalaza and the micropyle lie in a line, the hilum a little to one side of the micropyle.

The endosperm is massive, with a deep groove on one side into which fits a projection of the lignified coat. The cells are large thin-walled ones containing protoplasm packed with both protein reserve bodies (with globoid-like inclusions) and numerous small globules of oil, but no starch grains. The oil content tests 51 per cent. This region gives a striking phytosterol test.

The embryo is extremely small, being 1 mm. in length and 0.4 mm. in diameter. In the freshly gathered seed it shows the same degree of differentiation as it does when the coat splits preliminary to germination, although at this stage it has almost doubled in dimensions. It consists of a hypocotyl and two leaflike cotyledons between which lies a mass of undifferentiated cells, the plumule.

In the germinating seed the shift in the rate of growth is about as

follows. The cotyledons invade the endosperm and extend approximately throughout the length of it before the hypocotyl emerges. In this process one cotyledon usually increases in size more rapidly than the other. Cell contents and cell walls disappear as these cotyledons grow. Soon the hypocotyl takes the lead. First the basal portion pushes out through the endosperm and nucellus; then the upper portion grows rapidly and arches upward, pulling the cotyledons from the remnant of the endosperm. Regularly by this time four secondary roots have appeared in the transition region between root and stem. The cotyledons remain on the plant for four months or more, by which time the plant has acquired seven or eight other leaves.

An interesting change in correlation occurred in 40 per cent of the seedlings developing from seeds previously treated with NaCNS. The upper portion of the hypocotyl pulled the cotyledons out of the endosperm before the emergence of the lower end of the hypocotyl.

Germination tests: Positive results

Tables I and II indicate the type of results with 1929 seed. Each shows the general increase in percentage germination given by these seeds when provided the temperature alternation of 10° and 23°C. The various compounds used in the attempt to increase the percentages of germination or decrease the time required had no marked effect. With one exception the seeds germinated in as high percentage when provided distilled water. This result, the 50 per cent germination secured in one culture with N/5000 zinc sulphate, was probably a chance one as it could not be repeated. No germination was secured in the presence of many compounds tried. A list of these is given.

The time required for germination of seeds with the lignified coats removed from one side at the highest temperature tried, 29°, was 12 days as compared with 30 days required at the alternation and at 20°C. The total percentage was greatest at the alternation.

The time required for germination at 20° or 23° is shortened by storage at 10° in moist sand for 6–10 weeks, whereas a continuance of this storage for 18 weeks seemed less effective, as then the seeds required a longer time to germinate at the same temperature. The increase in time required may be due to the lowering of vitality by

the $CaOCl_2$ used in disinfecting the seeds. Some of it remains in the chalazal region.

Seeds with the lignified coats on when set to germinate at 23° required nine weeks; those with this coat off on one side required from three to four weeks at the same temperature.

TABLE I

PERCENTAGE GERMINATION GIVEN BY SEEDS OF LOT 2 (SEEDS RECEIVED IN PULP; PREVIOUS STORAGE CONDITIONS NOT KNOWN, BUT PROBABLY DRY AT AIR TEMPERATURE FOR TWO MONTHS)

STORAGE CONDITIONS AFTER RECEIPT	TEMPERATURE AT WHICH SET TO GERMINATE	MEDIUM	LIGNIFIED COAT ON OR OFF ON ONE SIDE	PERCENTAGE GERMINATION
10°, moist sand for 30 days..	Alternation of 10° and 23°	N/100 KNO_3	Off	15
Dry at room temperature for 30 days.....	Alternation of 10° and 23°	H_2O	"	30
Dry at room temperature for 30 days.....	Alternation of 10° and 23°	H_2O	"	25
Dry at room temperature for 30 days.....	Alternation of 10° and 23°	H_2O	"	35
Dry at room temperature for 30 days.....	23°	N/2500 $MnSO_4$	"	10
Dry at room temperature for 30 days.....	"	H_2O	"	5
10°, moist sand for 18 days	"	N/5000 $ZnSO_4$	"	10
" " " " " " ..	"	H_2O	"	15
" " " " 24 " ..	"	H_2O_2	"	5
Dry for time.....	"	1% NaCNS	"	15
10°, moist sand for 24 days..	"	N/5000 $ZnSO_4$	"	10
" " " " " " ..	"	" "	"	15
Dry.....	"	" "	"	5
"	"	H_2O	"	5
10°, moist sand for 38 days..	"	N/5000 $ZnSO_4$	"	10
" " " " 43 " ..	"	H_2O	"	5
" " " " 18 " ..	20°	H_2O	On	10
" " " " 38 " ..	"	H_2O	"	25
" " " " 18 " ..	"	N/5000 $ZnSO_4$	Off	15
" " " " 40 " ..	"	N/500 $ZnSO_4$	"	10

Seeds stored in moist sand at about 10° started germinating in 16 weeks and continued this for eight more weeks, when the number began decreasing. Those planted in garden soil in the greenhouse at

a temperature of about 25°C. within a week after being gathered began germinating in 14 weeks.

TABLE II

PERCENTAGE GERMINATION GIVEN BY SEEDS OF LOT 3

STORAGE CONDITIONS	TEMPERATURE AT WHICH SET TO GERMINATE	MEDIUM	LIGNIFIED COAT ON OR OFF ON ONE SIDE	PERCENTAGE GERMINATION
Dry at room temperature for 18 days then in moist petri dishes at 10° for 80 days..	Alternation of 10° and 23°	H_2O	Off	30
Dry at room temperature for 18 days then in moist petri dishes at 10° for 80 days..	Alternation of 10° and 23°	H_2O	"	40
Dry at room temperature for 18 days then in moist petri dishes at 10° for 80 days..	Alternation of 10° and 23°	N/5000 $ZnSO_4$	"	20
Dry at room temperature for 18 days then in moist petri dishes at 10° for 80 days..	Alternation of 10° and 23°	NaCNS	"	25
Dry at room temperature for 18 days then in moist petri dishes at 10° for 80 days..	Alternation of 10° and 23°	H_2O	On	35
Dry at room temperature for 18 days then in moist petri dishes at 10° for 80 days..	Alternation of 10° and 23°	H_2O	Off	40
Dry at room temperature for 18 days then in moist petri dishes at 10° for 80 days..	23°	H_2O	"	30
Dry at room temperature 100 days.....	Alternation of 10° and 23°	H_2O	"	35
Dry for 18 days.....	29°	H_2O	"	10
" " " "	"	H_2O	"	20
" " " "	"	N/800 butyric acid	"	15
" " " "	"	N/1000 acetic acid	"	10
" " 32 "	"	N/500 $ZnSO_4$	"	10
" " 39 "	"	H_2O	"	10
" " " "	"	H_2O	"	10
" " 45 "	"	N/5000 $ZnSO_4$	"	50
" " " "	"	N/100 $NaNO_3$	"	5
" " " "	"	1% NaCNS	"	10
" " " "	"	2% ethylene chlorhydrin	"	5

Germination tests: Negative results

Seeds stratified in moist sand were subjected for 48 hours to a temperature below freezing which caused grains of sand to cling lightly together. All seeds whether their coats were already split or still intact were killed.

Lots 1 and 4 did not germinate at all. Both were apparently sound. The behavior of each differed when stratified and again when set to germinate at 23° from that of lots 2 and 3. Lot 1, the 1928 seed, absorbed less water, 31.2 per cent as compared with 39 per cent absorbed by lot 3. Upon the seeds of lot 4, seeds received from a dealer seven months after they were gathered, blister-like elevations formed, raising the nucellus. These burst after a day or two, liberating a fluid giving the Flückiger test for reducing sugars. Disintegration of the mealy endosperm followed quickly.

Seeds of lot 1 were tested on cellucotton moistened with N/10 HCl, N/10 $HC_2H_3O_2$, N/500 $ZnSO_4$, N/100 butyric acid, N/1000 acetic acid, N/3400 HCl, and 10 per cent dioxygen. Seeds of lots 2 and 3, with lignified coats on or off, gave negative results on N/3400 and N/6800 HCl, N/1600 butyric acid, N/2000 acetic acid, N/5000 $MnSO_4$, 5 per cent KCl, 1.5 per cent acid monopotassium phosphate, 5 per cent glucose, Knop's nutrient solution, sterilized extract of the outer coat, and castor bean lipase plus N/1000 acetic acid. These same seeds failed to respond to soaking for 30 minutes in these reagents: 1 per cent thiourea, 2 per cent KCNS, 2 per cent chloral hydrate, and chloroform in the dilution of one drop in 100 cc. of H_2O.

Embryos removed from the endosperm of dry seeds did not grow on glucose, Knop's solution, sterilized extract of the outer coat, 2 per cent dioxygen, or distilled water.

Chemical changes

Reducing sugars.—In the dry seed there is a trace of glucose in the endosperm directly surrounding the embryo, in the distal half of each cotyledon, and throughout the epidermis of the cotyledons. As the coats split, the first noticeable increase is in the endosperm around the whole embryo. As the seed reaches the stage when the cotyledons are actively invading the endosperm, reducing sugars become more abundant in the periphery of the embryo and occur also

in the plumule and in the cells near the vascular tissues of the hypocotyl. At this stage there is a trace of glucose throughout the endosperm and enough present in some cells near the embryo to give ready formation of osazones. At the time of withdrawal of the cotyledons from the remnant of endosperm, reducing sugars are abundant in the phloem and endodermis of the root and stem, and are present throughout the seedling. In this remnant of the endosperm there is a considerable quantity of reducing sugar, together with much oil and a little protein. Reducing sugars are therefore present in both endosperm and embryo of the dry seed. As the seed prepares for germination these substances increase in both regions, notably in the endosperm near the embryo, in the epidermis of the embryo, in the cotyledons, and in meristem in general. No mashing or packing of cell walls of depleted cells takes place; the walls are digested.

SUCROSE.—Sucrose was found in a few instances with the glucose, but in very small quantities. In all tests greater quantities were found when the hydrolyzing agent was citric acid than when it was invertase. This may indicate the presence of glucosides which were hydrolyzed by the acid but were not affected by the invertase.

STARCH.—Starch was not found in either the endosperm or in the embryo of a dry seed. It was not present in one stratified in damp sand at a temperature of about 10° for 14 weeks or in one with coats barely split. It appears in the mesophyll of the cotyledons as they begin their growth, however, and increases rapidly in amount. Soon the cotyledons become packed throughout, with the exception of an area at the tip and the epidermal cells where none ever appeared. At this stage in some seeds both the endosperm cells lying between the cotyledons of the embryo and others touching it contained starch grains. Before the hypocotyl has pushed out, it also shows considerable starch except in cells of the growing point. It occurs abundantly and in large aggregates of grains in the pith, in pith rays, and in the region of the pericycle and endodermis. Scattered small grains of starch were present everywhere throughout the cortex. By the time the hypocotyl has extended the length of the seed, starch has increased throughout the embryo and become noticeable in some epidermal cells. Root hairs exhibit groups but all growing points are free of them. Starch remains abundant in the embryo until the up-

per part of the hypocotyl begins the rapid growth and arching which draws the cotyledons out from the small remnant of endosperm; then it begins decreasing. At the time of withdrawal of the cotyledons it is present in small quantities in the pericycle, endodermis, mesophyll of cotyledons along veins, and all tissues just below the plumule.

Oil.—There is a great amount of oil in the seed of *Magnolia grandiflora*, 51 per cent on the basis of vacuum-oven dry weight of the seed with coat removed. It is present in both endosperm and embryo in all stages studied, even in those cells of the endosperm touching the embryo, which in the dry seed were found almost free of protein reserves, presumably depleted by the embryo in its development. In the dry seed the oil tends to collect in large globules when mashed out of the cells. In the imbibed seed with coats barely split the oil exhibits the same characteristics and distribution as in the dry one. With the decided growth of the cotyledons, however, followed soon by the pushing out of the hypocotyl, changes in amount and size of the globules become apparent. There is a decrease of oil in the embryo, while in the endosperm it disappears slowly from the cells touching the embryo and a few rows beyond these. At this time there exists a gradation from cells next to the embryo in which there is only a small globule or none at all, through those with large globules, outward to cells with the whole protoplast crowded with them. In these cells which are almost free of oil the protein is completely gone while the cell wall is yet intact. It also finally disappears. Oil globules mashed out of such cells in which oil seems disappearing aggregate in much smaller globules than those from the endosperm of dry seeds, but are not of the size Pack (37) describes and considers a translocation form. Rhine (39), however, denies a translocation form of fat in the fatty seeds investigated. Oil is present in all cells but is not so abundant as in younger embryos, particularly in those of dry seeds. The decrease in oil seems correlated with the increase in starch and reducing sugars already noted.

Reserve proteins.—The endosperm cells in the dry grain, with the exception of an area about six cells wide around the embryo, are packed with protein grains, each containing a body which is colorless when treated with picronigrosin. In these cells the protein reserves occur in a decreased amount or are even almost absent in those cells

touching the embryo. Table III summarizes the tests applied to these grains of protein and the amino acid content of the proteins as indicated by them.

The bromine test for free tryptophane gave negative results.

The fewer granules in cells giving the test for soluble proteins are larger than in cells packed with them, and are also much less dense in appearance. In the endosperm of a seed with the cotyledons actively invading it, there is an increasing gradation outward in number of reserve protein grains parallel to that described for oil globules. This same area shows an inverse gradation with reference to soluble proteins.

TABLE III

TEST	AMINO ACID RESPONSIBLE FOR POSITIVE TEST
Xanthroproteic	Tryptophane, Tyrosin, Phenylalanine
Millon's	Tyrosin
Bromine	Histidine
Formaldehyde and H_2SO_4	Tryptophane

SOLUBLE PROTEINS.—By means of the Biuret test soluble proteins were demonstrated. They occurred in small quantities in all seeds tested, whether dry, imbibed, or germinating, and were found most conspicuous in amount in the cells near the embryo in which reserve proteins were scarce. As the seed coat splits, the embryo first shows the test throughout with a deeper color in the root tip, plumule, and epidermis of the whole embryo, and later in the phloem of the hypocotyl and throughout the cotyledons. At the time of emergence of the hypocotyl a striking test is exhibited by the whole of the remainder of the endosperm. The fact that the cotyledons give only a slight test is puzzling. As asparagin gives the Biuret test, these results can probably be interpreted from that standpoint also.

AMINO ACIDS AND ASPARAGIN.—No amino acids could be crystallized out of the dry seed. In tests on the germinating seed, leucine and asparagin were determined, together with an unidentified amino acid which formed broad needle-like crystals. Apparently there was an increase in these with increase in growth of the embryo. No attempt at localization of these acids was made. Quantitative studies could not be carried out because of scarcity of germinating seeds.

Reaction.—The endosperm of a seed with its coats split gave an alkaline reaction with neutral red; the embryo, a very slight acid reaction in the periphery. With further growth this acidity increases in the epidermis of the embryo and becomes noticeable in the end of the hypocotyl and in its vascular regions, as well as in a region of the endosperm surrounding the embryo. The plumule and the pith cells in the upper region of the hypocotyl are still alkaline in an embryo with its hypocotyl 1 cm. in length.

Tests for free acid in seeds ground with sand and transferred to boiled distilled water were made by titration with N/NaOH, using phenolphthalein as indicator. Table IV presents the data. Three seeds were used in each determination.

TABLE IV

Condition of seed	cc. of N/NaOH necessary to neutralize free acids present
Coats barely split	0.096
Coats widely split	0.289
Hypocotyls 1 cm. long	0.395

Other tests: Nitrates.—No evidence of nitrates could be secured in the tests on various stages studied. Seedlings with hypocotyls more than 1 cm. long were not available at the time these were run.

Iron.—Iron was demonstrated in both endosperm and embryo of dry seeds, and showed no change in distribution in one with coats split. In a seedling having the hypocotyl 8 cm. long a striking localization appeared in the root tips and in the cambium of the hypocotyl. Iron was present in the cotyledons but not in such quantities along the veins as could be expected from Molisch's results (32).

Silicon.—Tests with phenol crystals failed to show any silicon. This test was carried out in attempting to determine the composition of the heavy walls of the cortical parenchyma and pith of the seedlings.

Phytosterol.—The protoplasmic contents of the endosperm cells of all seeds gave a decided test for phytosterol with ether and H_2SO_4.

Enzymes and glutathione

Peroxidase and catalase occurred in small amounts in dry seeds and increased during germination. Oxidase, on the other hand, al-

though not indicated by the microchemical tests on either dry seeds or on those with the coats barely split, was most evident in later stages, appearing in the epidermis of the cotyledons and in the endosperm contiguous to them. Glutathione, however, is present throughout the endosperm of a seed with the coat split. Table V shows the distribution and quantity of glutathione in seeds and seedlings of

TABLE V

DISTRIBUTION OF GLUTATHIONE IN SEEDS AND SEEDLINGS OF MAGNOLIA GRANDIFLORA

MATERIAL	PERCENTAGE WATER CONTENT	COLOR GIVEN BY			
		HYPOCOTYL	COTYLEDONS	PLUMULE	ENDOSPERM
Dry seed	6	−	−	−	−
Soaked seed	37	−	−	−	+
Seed with coats barely split	49	+	−	−	+
Seed with coats well split but hypocotyl not protruding	61	+	+	−	++
Hypocotyls 1 cm. long	Not determined	++	−	−	+++
Hypocotyls 2 cm. long	Not determined	++	Veins ++	+	++++
Hypocotyls 5 cm. long	Not determined	+	+	+	++

Magnolia grandiflora, as indicated by the depth of color of the nitroprusside test.

Glutathione appears first in the lower end of the hypocotyl, then in the phloem of the vascular region. Its first appearance in the cotyledons is in the veins. When it is somewhat abundant in the hypocotyl in general it is present in greatest amounts just above the root cap and in the phloem regions. Parallel tests run on *Ricinus* seeds showed the same general distribution. The amount indicated in the meristem of the root and in the phloem, however, was far greater than in the reserves.

Table VI gives results of the quantitative determination of oxidase activity. Other tests should be run on the same types of materi-

al and on dry seeds without pyrocatechin in the apparatus to measure the autoxidative activity of substances present in them.

Table VII shows the catalase findings reduced to standard conditions. A range is given in each case showing the lowest amount

TABLE VI

OXIDASE DETERMINATIONS

MATERIAL	CC. OF O_2 REMOVED IN 10 HOURS MEASURED IN CC. HG
Six seeds with coats barely split	1.6
Six seeds with cotyledons invading endosperm	7.4
Reagents alone	0.2

TABLE VII

CATALASE ACTIVITY

MATERIAL	CC. OF O_2 LIBERATED FROM 10 CC. OF DIOXYGEN BY 3 SEEDS IN 10 MINUTES	
	LEAST AMOUNT DETERMINED	GREATEST AMOUNT DETERMINED
Dry grains soaked 24 hours	11.80	16.70
Dry grains which would not swell appreciably	3.90	9.40
Stored at 12° for 5 months but not germinating	7.79	10.85
Endosperm showing, seed split	16.70	24.67
Endosperm showing considerably	19.20	27.10
Hypocotyls barely emerging	48.36	54.20
Hypocotyls less than 1 cm. long	66.12	.....
Hypocotyls longer, 2–6 cm.	78.76	.....
Endosperms of dry soaked grains	11.35	14.80
Embryos of dry soaked grains	2.96	3.15
Endosperms of seed with coat barely split	13.80	18.75
Embryos of seed with coat barely split	2.96	5.92

found and the highest. No attempts were made to determine the localization of the catalase other than to discover whether it is present in both endosperm and embryo.

Peroxidase seemed localized in the embryo end of the seed in both the dry ones and those with the coats barely split. It, also, increases during early germination stages.

Discussion

Germination tests

The germination tests revealed very little of interest. The fact that seeds and young seedlings cannot endure a light freeze for 48 hours accounts for the narrow range of the tree in nature.

The cause of the delay in germination lies partly, but not wholly, in the lignified seed coat. Removal of this coat allows a low percentage of germination in three or four weeks' time, in seeds which require from two to three times as long with the coat on, or which do not germinate at all. Failure to germinate in such cases is due usually to death of the seed caused by invasion by fungi. Other causes for delay have not been determined. Whatever they may be, they are removed in many seeds by subjecting them to alternating temperatures. HARRINGTON (**23**) discusses the various theories put forward by others, and advances a theory of his own to explain the mechanism of breaking dormancy by alternating temperatures. He thinks that possibly at low temperature there may be an accumulation and actual metabolism of oxygen in a form which becomes available for inception of growth at higher temperatures. MORINAGA (**34, 33**) believes we have yet to learn the mechanism or mechanisms of the action of alternating temperatures and their substituting factors. His evidence seems to indicate that the effect is on the embryo. The effect of alternating temperatures on the germination of *Magnolia grandiflora* may be on the nucellus, on the endosperm, on the embryo, or on more than one of these structures. In this connection the 50 per cent reduction of gaseous exchange in *Ambrosia* seeds by the nucellus at 30°C. found by DAVIS (**11**) is of interest. He believes dormancy in seeds may be caused by the coats restricting the oxygen supply just below the amount requisite for germination at the temperature used. Moreover, he (**12**) induced dormancy in seeds of *Xanthium* by this method. In oily seeds probably oxygen is important in the formation from the fats of more or less unstable compounds which are usable by the embryo in germination.

None of the compounds reported by others as forcing agents had any decided value. It is not known whether or not they penetrated the nucellus. Interesting among these are the following: nitrates,

sulphates, and acetic acid which Rose (40) found to force *Sambucus* seed; dextrose and extract of the fleshy outer coat which Ives (27) found to force holly seeds; weak acids which Eckerson (17) found forced *Crataegus* seeds; KNO_3 and $MnSO_4$ for which Popoff (38) and others claim much; ethylene chlorhydrin, thiourea, and NaCNS found of value by Denny (13, 14) in breaking the dormancy of potatoes (possibly through inducing the production of oxidizing enzymes in greater quantity or of greater activity); increased oxygen supply secured through supplying H_2O_2 to the seed bed, as found valuable by Shull (42); and light as found stimulating by Gardner (21).

Why the seeds failed to germinate at all in the presence of some of the reagents is puzzling. In some cases their death was caused by rapid and vigorous growth of mold on the seed in presence of the reagents.

These studies on germination serve to point out the necessity of determining such matters as the permeability of the nucellus and of the endosperm cells to oxygen, CO_2, and various chemicals; the factors influencing the water-absorbing power of the endosperm and the embryo; the effect of other temperature alternations on the germination of freshly gathered seeds and on those variously stored, etc.

Chemical changes and oxidation systems

The chemical changes studied in seeds of *Magnolia grandiflora* are especially those occurring between the time of splitting of the seed coat and protrusion of the hypocotyl. The interval between setting to germinate and splitting of the coat was 16 weeks at 10°, and six weeks with alternation of temperature; that between splitting the coat and protrusion of the hypocotyl ranges from six days at 23° to 21 days at 10°C. During this latter interval the trend of both transformations and translocations taking place during the preparation for germination is the same as workers in this field have demonstrated for other seeds. The time required may differ greatly. Eckerson (17) expressed this idea clearly in stating that *Crataegus* goes through the same changes during the 90 days of after-ripening as are telescoped into the first eight days of germination of *Ricinus* as described by Deleano. Ives (27), also, says that the changes

during the long time needed for growth of the immature embryo of *Ilex opaca* are identical with those enumerated by PACK (35) as accompanying after-ripening in *Juniperus*. DAVIS (10) reported results similar to PACK's (35, 36). She thinks of after-ripening changes as the accumulation of readily usable materials such as sucrose, starch, amino acids, and soluble proteins, whose utilization by the seedling begins with germination and is accompanied by further breakdown of other less easily hydrolyzable reserves. In *M. grandiflora* the accumulation of the starch and soluble proteins in the embryo apparently follows the hydrolysis of reserve proteins and oil in the endosperm cells adjoining the embryo. Although the embryo itself possesses very meager reserves of its own upon which to draw, it seems slow in hydrolyzing its oil reserves. Soluble proteins and reducing sugars in slight quantities are present in it before there is evidence of active changes in the endosperm. It is to be noted, too, that the disappearance of reserve proteins and oil invariably begins next to the embryo, indicating possibly that the embryo somehow initiates these transformations. Questions to be answered here are, how does the embryo effect this, and what stimulates it to activity? These will be discussed later.

Of particular significance and interest are the observations on the disappearance of both protein and oil reserves. The protein bodies swell, become less dense, and disappear entirely. DAVIS (10) and LAKON (29) report similar changes in size of protein granules in storage cells of other seeds. In the cells depleted of protein granules, soluble proteins are strikingly indicated by the pinkish purple color obtained in the cells by the application of the test. Physiologically the seed of *Magnolia grandiflora* resembles that of *Fraxinus excelsior*, as described by LAKON (29), in that in each one a well differentiated embryo grows within the endosperm simultaneously with depletion of proteins stored there. LAKON calls this growth, this preparation for germination, "Vorkeimung," and states that it will occur in six months in moist sand. At the end of this time the seed germinates; that is, the hypocotyl emerges through the inclosing structures. Removing the coats did not overcome the dormancy. Starch appeared in the embryo within ten days after it was put in the sand, but was never present in the endosperm. The reserve proteins became muci-

laginous before disappearing. LAKON thinks they are glyco-proteins which give rise to carbohydrates in their breakdown, as well as to the commonly accepted protein decomposition products.

Clearly in *M. grandiflora* the soluble proteins are derived from the protein reserves. These shorter-chain protein compounds, on being hydrolyzed further to amino acids, pass into the embryo where resynthesis quickly occurs. BROWN and MORRIS (4) consider that the soluble proteins of barley seeds pass very early into the embryo as it swells, and serve there for the development of enzymes. This may be the situation in the seed of *M. grandiflora*, for certainly enzymatic activity of the epidermis of the embryo seems in some way awakened or greatly increased. Of course these proteolytic products of the reserve proteins serve as material for building protoplasm.

Fats also disappear from the endosperm cells adjoining the embryo. Great stores of starch appear in the mesophyll of the cotyledons as this happens. Cell walls also are being broken down. It seems strange that greater quantities of reducing sugar could not be demonstrated in the endosperm and in the cotyledons at this time. The fact that starch can be found in the endosperm cells now and then during disappearance of fat is interesting in this connection. *M. grandiflora* must have very active diastases. If the carbohydrate-like fragments of the fats, whatever they may be, are not used quickly as respiratory or building materials, they seem almost immediately to be condensed and stored as starch. Starch appears later in other parts of the embryo and is always present near growing points but not in them. PACK (36) thinks that in *Juniperus* both fats and proteins contribute to the carbohydrates found.

The acidity accompanying hydrolysis of fats and proteins in *M. grandiflora* can hardly be related in a causal way to the digestion of both, for, in general, lipase has its optimum pH range from 4.0 to 8.6; and plant trypsins from 7.0 to 9.5. Yet it must be remembered that pepsins have an acid optimum range, and that it is short-chain proteins that are abundant and not amino acids. The acidity is probably due to the fatty acids released. Increases in acidity during the germination of oily seeds have been reported time and again, notably by GREEN (22) who discovered lipase, by MILLER (31) in

his work on sunflower seeds, and by IVANOW (26) and ECKERSON (17).

No accumulation of reducing sugar directly precedes growth in an organ during the development of seedlings of *M. grandiflora*. TOOLE (43) reports a correlation between the appearance of reducing sugar in a tissue or organ and its decided enlargement. ECKERSON (18), CHOATE (8), PACK (35), and DAVIS (10) report much reducing sugar present in the hypocotyl or coleorhiza just before the pushing out of the hypocotyl. In *M. grandiflora* reducing sugars can be demonstrated in growing regions but never in large amounts. There seems to be no correlation in time of appearance with the swelling or growth of the organ.

The presence and development of the oxidizing systems is worthy of some comment. The presence of catalase in dry seeds and its increase during the preparation for germination and during the process itself are in agreement with the findings of many investigators. There are, however, notable exceptions. The significance to be attached to the catalase activity of the seeds depends entirely on the function assigned to it. If it is even a rough measure of metabolic activity the increase is significant. Catalase is present in both endosperm and embryo of the seed of *M. grandiflora*.

Although the oxygenase component of oxidase was not indicated by microchemical tests on dry seeds or on those with coats split, it did develop before the emergence of the hypocotyl. ECKERSON (17) reports late appearance of oxidase in seeds of *Crataegus*. TOOLE (43) did not find oxidase in seeds or seedlings of maize. ROSE (40) reports an increase in *Tilia* seeds on germination. Peroxidases, on the other hand, are usually considered to be present in seeds.

As oxygenase was found to be lacking in seeds of *Magnolia grandiflora* which had split their seed coats in preparation for germination, and which must have been respiring, tests for glutathione were made. It was absent in dry seeds but present in all later stages of germination, increasing with development, particularly in growing points and in the phloem until the hypocotyl reached the length of 1 cm. Then it was found decreasing. Tests on *Ricinus* seeds gave similar results. The appearance of glutathione in both these seeds on soaking is in line with the observations of VIVARIO and LE CLOUX

given earlier in this paper. Its distribution in both meristem and reserves agrees with KOZLOWSKI's observations (28).

The presence of glutathione in a seed when oxygenase is absent is interesting in view of the fact that it has been considered the most important autoxidizable component of animal cells by eminent physiologists. Possibly other seeds reported as lacking oxidase may possess some such oxidation mechanism. It is significant, too, that iron is found throughout the seed, for late investigations on glutathione accept the idea that iron is a necessary part of the glutathione mechanism.

The fact that glutathione was demonstrated in seeds with split coats hints at the mechanism of absorption of O_2 in the Bunzell oxidase determination on seeds in this stage of development when microchemical tests indicated no oxygenase present. It reconciles the Bunzell determination and microchemical ones.

The sequence of changes during the preparation for germination raises many fundamental questions regarding coordinations of the processes occurring, their concatenations and articulations. One such point is the method of depletion of the endosperm. Does the embryo secrete extracellular enzymes, lipases, ereptases, diastases, to which the living endosperm cells are permeable; or are these enzymes preceded by proteolytic ones produced by the embryo which by killing the cells opens them readily to attack by others? Is it possible that the effect of the stimulated embryo is enzymatic in another sense, in that it removes the products of autohydrolyses already going on in the endosperm cells adjoining it and thus allows their continuance? Many endosperms are capable of self-depletion. Oily endosperms are supposed to be particularly so as they are considered to remain alive throughout artificial or natural evacuation. BRUSCHI (5) claims this for the endosperm of *Ricinus* and considers active respiration a necessary condition.

How could the absorption of water accelerate or start these changes in the endosperm? Glutathione appears with the entry of water in the endosperm of *Magnolia grandiflora*. VIVARIO and LE CLOUX consider that in peas it arises from the hydrolysis of polypeptide complexes. The water imbibed may serve to affect the cells in some way so as to bring about the production of glutathione.

Possibly it only modifies the permeability of the cell so that the necessary proteolytic enzyme already present can reach its substrate in the cell. With the production of glutathione, increased respiration is possible if oxygen is available. Do changes in the embryo condition the absorption of water by it, or does the water result as a by-product of increased respiration made possible by the development of the glutathione mechanism?

How do the after-ripening processes in *Magnolia grandiflora* differ from those described for many other seeds? The preparation for germination includes the absorption from the endosperm of a great amount of carbohydrates and products of protein hydrolysis, a process usually reported as taking place in many embryos after the hypocotyl protrudes. This is not strange, for the embryo of this species has very slight reserves and seems slow to attack its oil. The after-ripening includes also a fivefold increase in size of cotyledons, the principal absorbing organs of this embryo.

Summary

1. This paper deals with the behavior of seeds of *Magnolia grandiflora* during preparation for germination and the early stages of the process, with particular reference to the overcoming of delayed germination, to the chemical changes taking place during after-ripening, and to the presence and development of oxidizing mechanisms.

2. Dry storage in the pulp at room temperature seemed almost as advantageous as moist storage of the disinfected seeds at 10° C.

3. Seeds with the fleshy outer coat retained when set to germinate were destroyed by fungi. It was necessary to remove this coat and soak the seeds in $CaOCl_2$ solution in order to control this situation.

4. The lignified coat delays germination but does not prevent it in 5–20 per cent of the seeds. Alternation of temperature was the only means found of securing consistent germination percentages above the one just mentioned.

5. During the preparation for germination the seeds acquire an increased resistance to fungal attack.

6. The endosperm of a dry seed is composed of thin-walled cells containing protoplasm packed with small oil globules and grains of

protein reserves but no starch. Traces of reducing sugars and soluble proteins can be demonstrated in cells near the embryo.

7. The embryo in a dry seed has oil globules in every cell. It shows no protein granules nor starch grains, but a trace of reducing sugar and short-chain proteins.

8. The seed at the time of splitting of the coat has an embryo twice the dimensions of that of the dry seed, but still very small, being on the average only 2 mm. long and 1 mm. wide, lying in an endosperm 10 mm. long and 4 mm. wide.

9. Before the hypocotyl protrudes the cotyledons grow until they are almost as long as the endosperm within which they lie. Their width increases in the same proportion.

10. The chemical changes taking place between the splitting of the coat and the protrusion of the hypocotyl are in general trend the same as have been reported by others as occurring during after-ripening and germination. After-ripening in the embryo includes absorption of food from the endosperm, a process usually reported as occurring during germination but necessitated earlier in *Magnolia grandiflora* by the slight amount of reserve food in the embryo. During the interval just mentioned the water content increases from 49 to 61 per cent.

11. Oils decrease in amount in the endosperm, the process beginning near the embryo.

12. Soluble proteins increase in endosperm cells near the embryo and in the embryo itself when reserve proteins disappear in these endosperm cells.

13. Starch appears in the cotyledons in striking quantity when the reserves of the endosperm begin disappearing. No detectable amount of oil in the embryo disappears previous to the appearance of the starch.

14. Traces of reducing sugars as well as of soluble proteins occur in the dry seed. Increases in these in the embryo as germination begins cannot be correlated in time with the beginning of rapid growth of these organs.

15. Iron is present in all parts of the seed and abundant in root tips of the seedling.

16. The epidermal cells of the embryo increase in acidity as indicated by depth of color with neutral red; and the contents of endosperm near these cells change from alkaline to acid.

17. Oxidase is not demonstrated by microchemical tests in seeds with the coat barely split, but becomes evident before protrusion of the hypocotyl and increases during later development.

18. Glutathione appears in the seed during soaking in H_2O. It is demonstrable first in the endosperm, then throughout the embryo as germination progresses. It is present previous to development of the oxidase mechanism.

19. Peroxidase and catalase are present in the dry seed, increasing with germination.

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